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Office européen des brevets

(11) Publication number:

**0 093 851**  
**A2**

(12)

# EUROPEAN PATENT APPLICATION

(21) Application number: 83102392.4

(51) Int. Cl.<sup>3</sup>: **A 61 K 37/02, A 61 K 39/00,**  
**C 07 C 103/52**

(22) Date of filing: 11.03.83

(23) Priority: 15.03.82 US 358150

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(24) Date of publication of application: 16.11.83  
Bulletin 83/46

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(25) Designated Contracting States: AT BE CH DE FR GB IT  
LI NL SE

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(54) Fatty acid carriers for synthetic vaccines.

(57) A synthetic vaccine is contemplated comprising a peptide residue coupled to one or more alkyl or alkenyl groups of at least 12 carbon atoms or other lipophilic substance wherein said peptide residue contains a sequence of 6 amino acids corresponding to the sequence of such amino acids in a protein antigen or allergen where the greatest local average hydrophilicity of the antigen or allergen is found.

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BACKGROUND OF THE INVENTIONCROSS REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of each of copending applications Serial No. 223,558 filed January 9, 1981 and Serial No. 272,855 filed June 12, 1981, assigned to the assignee hereof, the disclosures of which are hereby specifically incorporated herein by reference.

Field of the Invention

This invention is directed to a synthetic vaccine, to a composition useful in eliciting formation of antibodies in a host animal and to a composition useful as a diagnostic aid. More especially this invention is directed to a synthetic antigenic composition comprising a synthetic peptide and carrier. Still more especially this invention is directed specifically to the nature of the carrier for the synthetic peptide.

DISCUSSION OF RELATED APPLICATIONS

In my co-pending applications referred to above I disclosed a new system for determining that portion of the protein of a natural antigen or allergen which is responsible for the antigenicity or allergenicity of the protein. More especially I defined a process for determining the specific sequences of amino acid of proteinaceous allergens or antigens which are causative of an immune response when compositions containing the same are injected into host animals.

Thus I disclose not only that method for determining the specific sequence of amino acids but a method of preparing synthetic antigens or allergens knowing the precise number and sequence of amino acids which must be present. I also

disclose numerous synthetic vaccines comprising a short polypeptide supported on a carrier, the carrier considered to be of critical importance in providing the active portion of the synthetic peptide chain with sufficient size so that the entire synthetic antigen or synthetic allergen can be recognized by the immune system and evoke formation of the corresponding antibodies.

Specifically, my synthetic vaccine comprises a physiologically acceptable carrier in or on which is disposed a synthetic peptide residue containing a sequence of at least six amino acids corresponding to the sequence of such amino acids in a protein antigen or allergen with the greatest local average hydrophilicity of the antigen or allergen, said local hydrophilicity of said protein antigen or allergen being defined by and determined by:

A. assigning relative hydrophilicity values to the amino acids of the protein antigen or allergen in accordance with relative relationship of such amino acids as shown in the table below:

TABLE 1.

<u>Amino Acid</u>	<u>Hydrophilicity Value</u>
Arginine	3.0
Aspartic Acid	$3.0 \pm 1$
Glutamic Acid	$3.0 \pm 1$
Lysine	3.0
Serine	0.3
Asparagine	0.2
Glutamine	0.2-
Glycine	0.0
Proline	$-0.5 \pm 1$

TABLE 1 (con't)

<u>Amino Acid</u>	<u>Hydrophilicity Value</u>
Threonine	-0.4
Alanine	-0.5
Histidine	-0.5
Cysteine	-1.0
Methionine	-1.3
Valine	-1.5
Isoleucine	-1.8
Leucine	-1.8
Tyrosine	-2.3
Phenylalanine	-2.5
Tryptophan	-3.4

B. determining the repetitive local average of hydrophilicity values at a plurality of points along the amino acid sequence:

C. determining from such local points of repetitive averages the points of greatest local average hydrophilicity; said composition being characterized by evoking a protective immunological response or by stimulation of antibody formation or decreased sensitivity to allergen when introduced into a host animal in the absence of the entire amino acid sequence of the protein antigen or allergen.

At the heart of the development there is the determination of a sequence of six amino acids which are critical to the production of the immunological response. In accordance with such earlier invention this is done with the foreknowledge of the amino acid sequence of an antigen or

1 allergen, but if the same is unknown, then the amino acid sequence  
2 of the entire protein must first be determined. This can be  
3 done by known but laborious means.  
4

5  
6 Given the amino acid sequence of the entire protein  
7 antigen or allergen, the next objective is to determine the  
8 point along said molecule where there is greatest local average  
9 hydrophilicity. This is initially done by assigning relative  
10 hydrophilicity values in accordance with the table above to  
11 each amino acid in the protein. Thereafter, those values are  
12 repetitively averaged along the length of the protein. While  
13 such method is partially successful (working for some proteins,  
14 but not others) when averaging groups range in size from four  
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- 5 -

1 to ten successively connected amino acids, it is preferred that in determining such local averages the hydrophilicity values of five to seven linearly connected amino acids be employed, especially six such amino acids. At a plurality of 5 points along the amino acid chain of the protein, the local averages are determined (moving average, increment of one).

Once the repetitive local averages of the specific hydrophilicity values are determined, the precise point of greatest 10 hydrophilicity can be easily located by inspection or determined graphically or otherwise. It has been discovered that the six amino acids providing the greatest local average hydrophilicity are the sequence of six amino acids which are critical to the production of the immunological response. 15 Stated differently, it has been found that this sequence of six amino acids is present in an epitope of the protein, i.e. the sequence of amino acids recognized by and bound by an antibody with immunological specificity. Such epitope is hereinafter designated as the "H-epitope" as it is the 20 epitope of greatest local average hydrophilicity.

With this realization of the precise sequence of amino acids which accounts of H-epitope of a given protein antigen or allergen, one can form a synthetic vaccine in any number of 25 ways.

The synthetic vaccine is prepared either by chemically synthesizing a chain of amino acids corresponding to the sequence of amino acids of the H-epitope or the H-epitope is 30 obtained from a protein containing the same by selective lysis

1 such as by splitting the protein by the use of enzymes. The  
2 amino acid chain containing the H-epitope so obtained either  
3 synthetically or from naturally occurring protein is thereafter  
4 disposed on a physiologically acceptable carrier, and the  
5 resultant composition is thereafter diluted with physiologically  
6 acceptable medium. The composition is then ready for introduc-  
7 tion into a host animal.

8  
9 It will be realized that the process of the invention  
10 is useful in the formation of synthetic vaccines of known and  
11 unknown, identified or unidentified, protein antigens or  
12 allergens, since the focus is upon the portion of the protein  
13 molecule which provides the H-epitope. Thus, the synthetic  
14 vaccine of the invention can contain H-epitopes of single or  
15 multiple known or unknown protein antigens or allergens. The  
16 synthetic vaccine can contain a plurality of H-epitopes of a  
17 single antigen or can contain a single H-epitope of a first  
18 antigen and an H-epitope of a second antigen or allergen. The  
19 synthetic vaccine can contain one or more H-epitopes of an  
20 antigen or allergen alone or in combination with one or more  
21 H-epitopes of a second antigen or allergen. In fact, the  
22 synthetic vaccine can contain as many epitopes corresponding  
23 to said sequence of six amino acids of greatest local average  
24 hydrophilicity as desired, and said epitopes can correspond to  
25 the sequence of six amino acids from a wide variety of antigens  
26 or allergens. The vaccine contains at least one H-epitope.  
27 This H-epitope can be co-present with other epitopes of the  
28 same or different antigens which are not H-epitopes, i.e., do  
29 not correspond to the point of greatest local average  
30 hydrophilicity of the antigen or allergen.

1 The process of the invention is useful in the formation of  
synthetic vaccines from antigens whose amino acids sequence:  
has not heretofore been reported. The art well knows how to  
determine the amino acid sequence of a protein antigen or  
5 allergen. It remains, therefore, a simple matter in accordance  
with the invention to determine the H-epitope.

The synthetic vaccine can have H-epitopes of any protein  
antigen or allergen. The vaccine of the following protein  
10 antigens or allergens are particularly contemplated: Hepatitis  
B surface antigen, histocompatibility antigens, influenza  
hemagglutinin, fowl plague virus hemagglutinin, rag weed  
allergens Ra3 and Ra5 and the antigens of the following  
viruses: vaccinia, Epstein-Barr virus, polio, rubella, cyto-  
15 megalovirus, small pox, herpes simplex types I and II,  
yellow fever, and many others.

It can also alternatively or additionally have an H-epitope  
of a protein of any of the following parasites: organisms  
20 carrying malaria (*P. Falciporum*, *P. Ovace* etc.), Schistosomiasis,  
*Onchocerca Volvulus* and filarial parasites, Trypanosomes,  
*Leishmania*, Chagas disease, amoebiasis, hookworm,  
and the like. In addition, vaccines of the following bacteria  
are especially contemplated: leprosy, tuberculosis, syphilis,  
25 gonorrhea and the like.

Vaccines of the following viruses can be made by the process  
of the invention: Infectious ectromelia virus, Cowpox virus,  
Herpes simplex virus, Infectious bovine rhinotracheitis  
30 virus, Equine rhinopneumonitis (equine abortion) virus,  
Malignant catarrh virus of cattle, Feline rhinotracheitis  
virus, Canine herpesvirus, Epstein-Barr virus (ass. with



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- 1 infectious mononuclosis and Burkett lymphoma), Marek's disease virus, Sheep pulmonary ademomatosis (Jaagziekte) virus, Cytomegaloviruses, Adoenovirus group, Human papilloma virus, Feline panleucopaenia virus, Mink enteritis virus, African
- 5 horse sickness virus (9 serotypes), Blue tongue virus (12 serotypes), Infectious pancreatic necrosis virus of trout, Fowl sarcoma virus (various strains), Avian leukosis virus, visceral, Avian leukosis virus, erythroblastic, Avian leukosis virus, myeloblastic, Osteopetrosis virus, Newcastle
- 10 disease virus, Parainfluenza virus 1, Parainfluenza virus 2, Parainfluenza virus 3, Parainfluenza virus 4, Mumps virus, Turkey virus, CANADA/58, Canine distemper virus, Measles virus, Respiratory syncytial virus, Myxovirus, Type A viruses such as Human influenza viruses, e.g. Ao/PR8/34, A1/CAM/46,
- 15 and A2/Signapore/1/57; Fowl plague virus; Type B viruses e.g. B/Lee/40; Rabies virus, Eastern equine encephalitis virus; Venezuelan equine encephalitis virus; Western equine encephalitis virus; Yellow fever virus; Dengue type 1 virus (= type 6), Dengue type 2 virus (= type 5), Dengue type 3
- 20 virus, Dengue type 4 virus; Japanese encephalitis virus, Kyanasur Forest virus; Louping ill virus; Murray Valley encephalitis virus; Omsk haemorrhagic fever virus (types I and II); St. Louis encephalitis virus; Human rhinoviruses; Foot-and-mouth disease virus; Poliovirus type 1; Enterovirus
- 25 Polio 2, Enterovirus Polio 3; Avian infectious bronchitis virus; Human respiratory virus; Transmissible gastro-enteritis virus of swine; Lymphocytic choriomeningitis virus; Lassa virus; Machupo virus; Pichinde virus; Tacaribe virus; Papillomavirus.

30

Similarly, the synthetic vaccine can have an H-epitope

35

1 of any protein allergen such as the rag weed allergens.  
2

3 It is to be understood that the foregoing lists are  
4 not all-inclusive, but simply exemplary, since the heart of the  
5 invention resides in reliably and confidently predicting  
6 and determining the H-epitope.  
7

8 In forming a synthetic vaccine according to the earlier  
9 invention, it is preferred to insure that the epitope has the  
10 steric configuration to be recognized by an antibody; that the  
11 given sequence of 6 amino acids have bonded thereto as part of  
12 the amino acid chain at least three amino acids on either  
13 side thereof, these three adjacent amino acids serving as  
14 auxiliary acids to insure the stabilization of the  
15 epitope so that it is readily recognized by and neutralized  
16 by an antibody.  
17

18 In one of its simplest forms, that invention comprises  
19 a physiologically acceptable carrier on which is disposed a syn-  
20 thetic peptide residue of the designated epitope. This synthet-  
21 ic peptide residue has a chain length of minimally six amino ac-  
22 ids, preferably twelve amino acids (considering the three amino  
23 acids on either side thereof) and can contain an infinitely long  
24 chain of amino acids or their components, which can be charac-  
25 terized by the presence of other epitopes of the same or  
26 different antigen or allergen. Where it is free of such  
27 additional chain with or without such additional epitopes, it  
28  
29  
30

1 generally does not have an amino acid chain exceeding 50 amino  
2 acids. Where a short chain is desired containing the desired  
3 epitope, it preferably does not have an amino acid chain length  
4 greater than 40, more especially not greater than 30 and more  
5 particularly not greater than 20 amino acids. Optimally the  
6 peptide residue has an amino acid chain length of 12 to 18  
7 amino acids, preferably 12 to 15 amino acids, especially 12  
8 amino acids.

9  
10 In my earlier application I disclose numerous physio-  
11 logically acceptable carriers for the peptide residue including  
12 those which are animal, vegetable and mineral. Specifically  
13 disclosed carriers included segments of polyamino acid, poly-  
14 saccharides, polyamides, vinyl polymers, ester polymers, as  
15 well as proteins especially subclass hemoglobin, human serum  
16 proteins, tetanus toxoid.

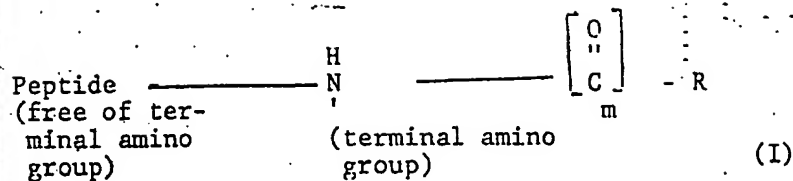
17  
18 One problem that exists in the field of vaccines re-  
19 lates to the nature of the carrier. Since optimally the vac-  
20 cine stimulates production of only those antibodies specific to  
21 the antigen or allergen, the carrier should not evoke antibody  
22 formation to itself. The production of antibodies or any  
23 other substance in response to the carrier portion of the vac-  
24 cine complicates the immune system's behavior, can be the  
25 cause of side reactions, and can compete with the production  
26 of antibodies to the synthetic antigen or allergen. It has  
27 therefore been desirable to provide a carrier for a synthetic  
28 vaccine where the carrier portion of the molecule is sub-  
29 stantially inert to the immune system and does not evoke the  
30 production of antibodies specific thereto. It is the  
31 further object of this invention to provide an improved

1 synthetic vaccine comprising a synthetic peptide residue  
2 disposed in or on a carrier where the carrier is one which  
3 is compatible with the organism into which the vaccine is  
4 to be introduced and can be readily metabolized by such host  
5 animal and in time be excreted without complications to the  
6 injection site or the various organs of the body.

#### 8 SUMMARY OF THE INVENTION

9 In accordance with this invention I provide an  
10 improved synthetic antigen or synthetic allergen of the type  
11 disclosed in the aforementioned applications for Letters  
12 Patent wherein the carrier is one comprising a straight or  
13 branched substituted or unsubstituted, saturated or unsaturated  
14 hydrocarbon residue of at least twelve carbon atoms. In  
15 particular, the carrier of the invention is one having at  
16 least twelve carbon atoms in a chain whose chain is either  
17 an alkyl or alkenyl group. Such alkyl or alkenyl group can  
18 have up to 36 carbon atoms but is preferably in the range of  
19  $C_{12}$  to  $C_{24}$ . These hydrocarbon residues can be provided by  
20 fatty acids by simple coupling of the fatty acid moiety to  
21 a terminal functional group of the synthetic peptide by  
22 relatively routine chemistry. I also contemplate, however,  
23 carrying the synthetic residue on such hydrocarbon residues  
24 without the use of the carboxylic acid functional group of  
25 the fatty acid whereby the synthetic peptide is joined to  
26 the hydrocarbon residue without a carbonyl connecting link.

27 Thus, my invention can be described broadly as a  
28 composition comprising a synthetic antigen or allergen of the  
29 formula:  
30



wherein

m is 0 or 1;

R is a substituted or unsubstituted alkyl or alkenyl radical of at least 12 carbon atoms; and

Peptide is a residue containing a sequence of 6 amino acids corresponding to the sequence of such amino acids in a protein antigen or allergen where the greatest local average hydrophilicity of the antigen or allergen is found, said local hydrophilicity of said protein antigen or allergen determined by:

A. assigning relative hydrophilicity values to the amino acids of the protein antigen or allergen in accordance with relative relationship of such amino acids as shown in the table below:

TABLE 1.

<u>Amino Acid</u>	<u>Hydrophilicity Value</u>
Arginine	3.0
Aspartic Acid	3.0 $\pm$ 1
Glutamic Acid	3.0 $\pm$ 1
Lysine	3.0
Serine	0.3
Asparagine	0.2
Glutamine	0.2
Glycine	0.0
Proline	-0.5 $\pm$ 1
Threonine	-0.4

TABLE 1. (con't)

<u>Amino Acid</u>	<u>Hydrophilicity Value</u>
Alanine	-0.5
Histidine	-0.5
Cysteine	-1.0
Methionine	-1.3
Valine	-1.5
Isoleucine	-1.8
Leucine	-1.8
Tyrosine	-2.3
Phenylalanine	-2.5
Tryptophan	-3.4

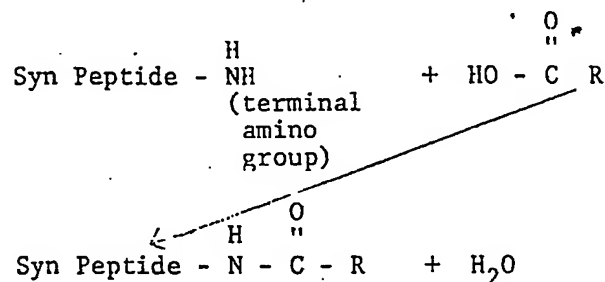
B. determining the repetitive local average of hydrophilicity values at a plurality of points along the amino acid sequence:

C. determining from such local points of repetitive averages the points of greatest local average hydrophilicity; said synthetic antigen or allergen when free of an amino acid sequence corresponding to the entire protein antigen or allergen evoking a protective immunological response or stimulating antibody formation for decreasing sensitivity to allergen when introduced into a host animal, in the absence of the entire amino acid sequence of the protein antigen or allergen.

Referring to the formula above I can couple the synthetic peptide moiety to an alkyl or alkenyl group of at least 12 carbon atoms by blocking all those amino groups of the synthetic peptide residue so that they are free of reactivity to a carboxylic acid except that a terminal amino

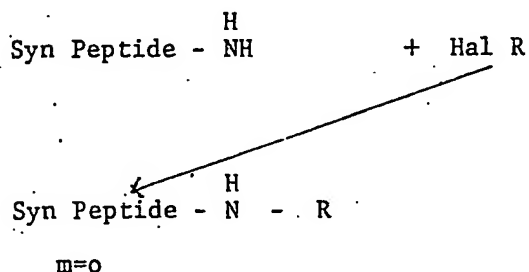
group remains available for reaction. I thereafter react the terminal amino group of the synthetic peptide with a moiety which supplies a carboxylic acid group whereby condensation of a hydrogen atom of the amino group with the hydroxyl group of carboxylic acid group (dehydration) effects interlinkage of the synthetic peptide with the carboxylic acid group in accordance with the following equation:

A.



In accordance with this reaction there is formed a composition as defined in equation I above wherein  $m = 1$ . I also envisage, however, disposing these synthetic peptides on a  $\text{C}_{12} - \text{C}_{36}$  alkyl or alkenyl moiety without using a carboxylic acid or similar functional group to link with the terminal amino group. Thus for instance I envisage a substitution reaction in accordance with the following equation:

B.



in which case there is formed a synthetic vaccine within formula I above wherein  $m$  is 0. Numerous alternative routes

- 15 -

1 to disposing a synthetic peptide on a  $C_{12}$  to  $C_{36}$  alkyl or  
2 alkenyl group are apparent; these invariably linking the  
3 synthetic peptide to the alkyl or alkenyl moieties via  
4 a terminal amino group of the synthetic peptide moiety.

5 In forming a synthetic vaccine in accordance with  
6 this invention, I prefer to use a fatty acid of  $C_{12}$  to  $C_{24}$ .  
7 Particularly contemplated fatty acids include the following:

8 Palmitic

9 Stearic

10 Behenic

11 Oleic

12 The Merrifield solid phase synthesis for synthetic  
13 peptides is a particularly desirable approach to formation of a  
14 fatty acid carried synthetic peptide, since it provides a  
15 convenient means for attachment of the carrier in accordance  
16 with the invention, although it should be understood that liquid  
17 phase approaches can also be employed. The Merrifield solid  
18 phase approach involves connecting amino acids to one another  
19 where the pendant reactive groups, e.g., amino, hydroxyl,  
20 carboxyl, imidazol groups, are blocked. After the final amino  
21 acid has been coupled, the N-terminus is deblocked and a fatty  
22 acid or other suitable large lipophilic substituent or com-  
23 ponent supplying a  $C_{12}$  to  $C_{36}$  alkyl or alkenyl group is re-  
24 acted by procedures outlined above for use in amino acid  
25 couplings, the procedure is carbodiimide mediated peptide  
26 (amide) bond formation, hydroxybenzotriazole ester addition  
27 or addition of a fatty acid symmetrical or asymmetrical an-  
28 hydride.

29 This results in a peptide with covalent N-terminal  
30 fatty acid or similar moiety. The peptide is then removed  
31 from the resin by typical hydrofluoric acid treatment, and



1 purified if necessary.

Such a fatty acid peptide conjugate is complete in and of itself and needs no additional carrier molecule or support  
 5 for immunological enhancement. It aggregates when placed in aqueous medium and aggregate is capable of stimulating a strong immune response similar to those achieved with carriers such as red blood cells or large proteins. Carriers of the invention, it is believed, are more suitable than carriers  
 10 such as red blood cells and large proteins as the latter carriers tend to prompt unwanted immune response directed against the carrier per se. Thus carriers of the invention provide effective and readily produced vaccines with minimal chance of unwanted immunological responses.

15

Particularly contemplated reactants include fatty acid anhydrides of the formula:



20

wherein

$\text{R}_1$  and  $\text{R}_2$  are independently alkyl or alkenyl, including alkenyl containing multiple unsaturation of several carbon atoms. However, symmetric saturated alkyl  $\text{R}_1$  and  $\text{R}_2$   
 25 groups are preferred. These fatty acid anhydrides react relatively readily with the peptide. Generally speaking, reaction is effected at a temperature of between 20° and 30° for between 2 and 4 hours. The reaction is performed in the presence of a solvent. Particularly contemplated solvents  
 30 include: methylene chloride, dimethylformamide or dimethylsulfoxide. Thereafter the peptide linked to the  $\text{C}_{12}$  to  $\text{C}_{36}$  alkyl <sup>or</sup> alkenyl group via amide moiety is removed from the Meerifield resin and the blocking agents are removed from the side chain groups by contacting the same with a

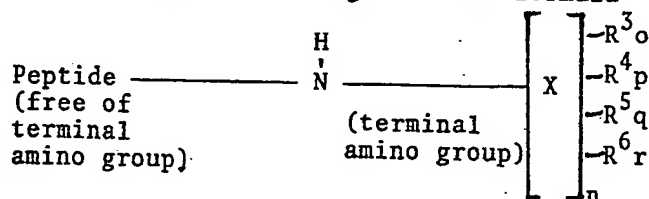
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1 strong acid such as: hydrofluoric, hydrobromic or methane-  
sulfonic acids. Thereafter the material is worked up in  
the usual manner or washed and the synthetic vaccine is  
recovered.

5

The peptide can be carried on a  $C_{12}^+$  alkyl or alkenyl  
caontaining carrier which comprises a plurality of  $C_{12}^+$   
alkyl or alkenyl moieties. Thus there is contemplated a  
synthetic antigen or allergen of the formula

10



15

wherein

$R^3$ ,  $R^4$ ,  $R^5$  and  $R^6$  are each  $C_{12}$ - $C_{36}$  alkyl or alkenyl  
groups which may be straight or branched chained and  
substituted or unsubstituted;

20

$o$ ,  $p$ ,  $q$  and  $r$  are each 0 or 1 and the sum of  $o$ ,  $p$ ,  
 $q$  and  $r$  is equal to  $n$ ;

$n$  is 2 to 4; and

$X$  is a polyfunctional group having 3 to 5 functional  
groups, at least one of which is bound to said

25

terminal amino group, and at least one of said  
functional groups bound to one of  $R^3$ ,  $R^4$ ,  $R^5$  or  $R^6$ .

Functional groups for  $X$  include carbonyl and amido.

The carbonyl group ist effective as a link to the terminal.

30

amino group whereby and amido group is formed while the

amido group is an effective link between the synthetic

peptide and the  $C_{12}$ - $C_{36}$  alkyl or alkenyl group.

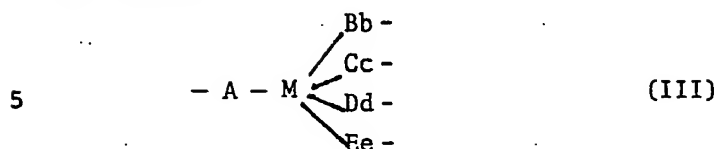
The functional groups can be separated by backbone

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which itself is an alkyl or alkenyl group, usually of chain

- 18 -

1 length of 2 to 5 carbon atoms. Thus X can be represented by  
the formula



wherein

- 10        A is a bifunctional group one end of which is linked  
to M and the other end of which is linked to the  
terminal amino group of the synthetic peptide;  
M is alkylene or alkenylene of 2 to 5 carbon atoms;  
B, C, D and E are bifunctional groups one end of  
which is linked to M and the other end of which is  
linked to a  $C_{12}-C_{36}$  alkyl or alkenyl group; and  
15        b, c, d and e are each 1 or 0 and the sum of b, c,  
d and e is 2 to 4.

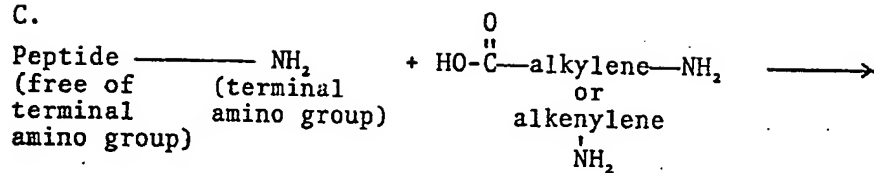
Specifically, such a structure can be provided using an  
amino acid having a plurality of amino groups. These amino  
20 groups are reactable with a source of  $C_{12}-C_{36}$  alkyl or alken-  
yl groups. The synthetic peptide's side chain reactive groups  
are blocked with, for instance, a blocking agent of the type  
described above. The terminal amino group of the synthetic  
peptide is reacted with the multifunctional group or amino  
25 acid whereby the peptide now is joined to a bridge having  
two or more reactive sites to which  $C_{12}-C_{36}$  alkyl or alkenyl  
groups can be bound. Thereafter the peptide-bridge inter-  
mediate structure is reacted with source of  $C_{12}-C_{36}$  alkyl or  
alkenyl groups.

30        The above embodiment can be described in terms of using an  
amino acid possessing a side chain and a terminal amino  
group.

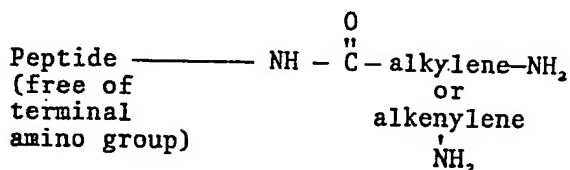
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1 C.



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10

The two free  $\text{-NH}_2$  groups remain reactive to source of  $\text{C}_{12}\text{-C}_{36}$  alkyl or alkylene groups. Usually the preparation of the intermediate involves the reaction of the terminal amino group of the peptide with such an amino acid where amino groups are protected by a functional group that is more readily removed than the side chain protecting groups of the peptide. Such protective but reactive groups include:

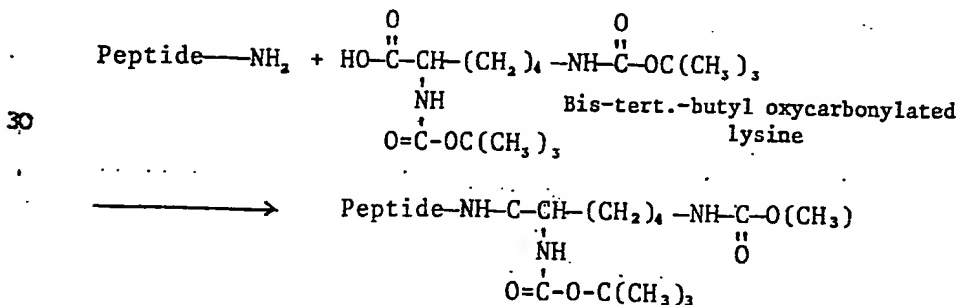
- Tertiary butyloxycarbonyl
- Trifluoroacetyl
- Fluorenylmethyloxycarbonyl

20

The preparation of these synthetic antigens or allergens containing multiple alkyl or alkenyl carriers is illustrated below for a synthetic peptide whose side chain groups are blocked:

25 blocked:

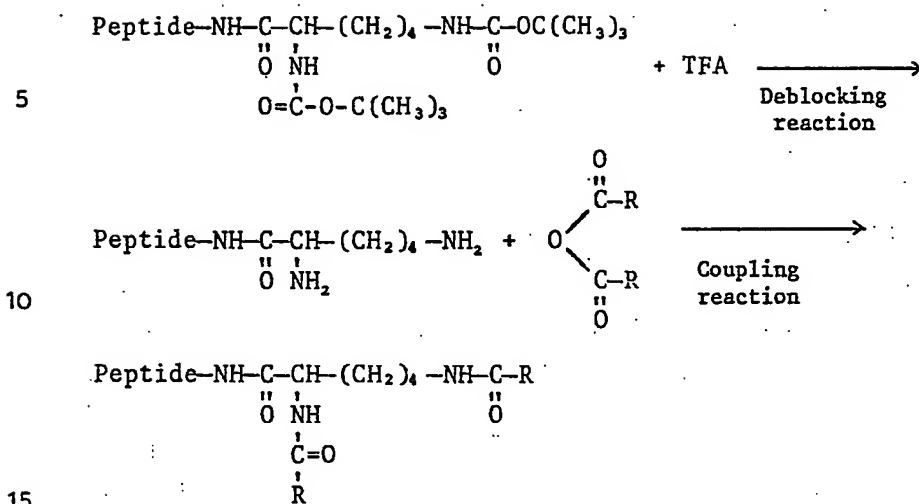
D.



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- 20 -

1 E.



TFA = trifluoroacetic acid

Of course, the R groups can be the same or different since mixed anhydrides are suitable reactants.

20

It should be understood that in the above set of equations a lysine backbone joined the respective functional groups. Essentially any link between functional groups is suitable although it is preferred to minimize the presence of those groups which would impart toxicity to the vaccine or stimulate or prompt production of antibodies specific thereto, it being the purpose to produce antibodies to the synthetic peptide.

30

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1 It is preferred that this link be an alkylene or alkenylene  
group, i.e. a bifunctional residue or radical of an alkane  
or alkene. These alkylene or alkenylene groups can be straight  
or branched chain and can have 2 to 6 carbon atoms.

5

#### DESCRIPTION OF SPECIFIC EMBODIMENTS

In the determination of the sequence of six amino acids  
which provide the H-epitope, it is preferred that more re-  
10 spective values than those set forth in the table below be  
assigned to respective amino acids in the protein antigen or  
allergen. Thus, there is set forth in the table below the  
broad, preferred and most preferred ranges to be assigned  
for the determination of six amino acids providing greatest  
15 local average hydrophilicity.

20

25

30

35

TABLE 2.

Amino Acid	Hydrophilicity Value		
	Broad	Preferred	Most Preferred
Arginine	3.0	3.0	3.0
Aspartic Acid	3.0 $\pm$ 1	3.0 $\pm$ .5	3.0
Glutamic Acid	3.0 $\pm$ 1	3.0 $\pm$ .5	3.0
Lysine	3.0	3.0	3.0
Serine	0.3	0.3	0.3
Asparagine	0.2	0.2	0.2
Glutamine	0.2	0.2	0.2
Glycine	0.0	0.0	0.0
Proline	-.5 $\pm$ 1	0.0 $\pm$ .5	0.0
Threonine	-0.4	-0.4	-0.4
Alanine	-0.5	-0.5	-0.5
Histidine	-0.5	-0.5	-0.5
Cysteine	-1.0	-1.0	-1.0
Methionine	-1.3	-1.3	-1.3
Valine	-1.5	-1.5	-1.5
Isoleucine	-1.8	-1.8	-1.8
Leucine	-1.8	-1.8	-1.8
Tyrosine	-2.3	-2.3	-2.3
Phenylalanine	-2.5	-2.5	-2.5
Tryptophan	-3.4	-3.4	-3.4

1 It will be recognized that these values are relative.  
2 By multiplying these values with a factor, one can obtain  
3 another set of values which can similarly be used to provide  
4 the same prediction and determination. The important concept  
5 is that the respective amino acids have the relative relation-  
6 ship as set forth in the table above. These arbitrary values  
7 are established for the purpose of providing a convenient  
8 means whereby the portion of a long chain protein molecule  
9 of highest hydrophilic characteristic is identified. When that  
10 is determined, the realization of the six amino acids accounting  
11 for that hydrophilicity peak is easily determined.  
12

13 Thus the procedure of the invention can be employed  
14 to determine the sequence of six amino acids which provide the  
15 most hydrophilic region of numerous unrelated antigens.  
16

17 Specifically, the hepatitis B surface antigen has  
18 been studied to determine the sequence of six amino acids which  
19 determine the H-epitope. The sequence of amino acids for  
20 such antigen is as follows:  
21

22 Lys Pro Thr Asp Gly Asn (which correspond to amino ac-  
23 ids 141-146 of the hepatitis B surface antigen protein). Simi-  
24 larly, the sequence of amino acids for the human histocompati-  
25 bility antigen HLA-B7 which determine the H-epitope is:  
26 Pro Arg Glu Glu Pro Arg (which correspond to amino acids  
27 43-48 of the protein).  
28

29 Similarly, the sequence of the amino acids for the  
30 influenza hemagglutinin antigen (X31 strain) which determine  
31 the H-epitope is: Val Glu Arg Ser Lys Ala (which correspond  
32 to amino acids 105-110 of the protein).



1       The H epitope for the A/memphis/102/72 strain of  
2 influenza hemagglutinin is: Lys Arg Gly Pro Asp Ser, corres-  
3 ponding to amino acids 140 to 145 of the protein.

4  
5       The H epitopes for two other strains of influenza  
6 hemagglutinin, A/Eng/878/69 and A/NT/60/68/29c, are identical  
7 to the H epitope of A/memphis/102/72 as stated above.

8  
9       The H epitopes of the A/NT/60/68 and A/Qu/7/70  
10 strains of hemagglutinin are identical and comprise the follow-  
11 ing amino acids: Arg Asn Val Pro Glu Lys corresponding to  
12 to amino acids 321 - 326 of the proteins.

13  
14       The H epitope for the neuraminidase protein of the  
15 A/PR/8/34 strain of influenza is Arg Gly Arg Pro Lys Glu Lys,  
16 corresponding to amino acids 413 to 419 of the protein. This  
17 epitope contains seven amino acids because it comprises two  
18 adjacent and overlapping H epitopes of equal hydrophilicity,  
19 as is the case for the Japan strain hemagglutinin already des-  
20 cribed (in the original manuscript).

21  
22       The H epitope for the diphtheria toxin fragment A  
23 is: Glu Thr Arg Gly Lys Arg, corresponding to amino acids 168  
24 to 173 of the protein.

25  
26       The H epitope for the avian sarcoma virus gp 37  
27 protein is: Leu Arg Glu Ile Glu Arg Leu, corresponding to  
28 amino acids 37 to 43 of the protein (again, two adjacent and  
29 overlapping H epitopes yielding a seven amino acid sequence).

30  
      The H epitope for the avian sarcoma virus src gene  
protein is: Lys Ser Lys Pro Lys Asp, corresponding to amino  
acids 5 to 10 of the protein.

1 The H epitope for the E3/16 protein (external portion)  
2 of the adenovirus type 2 strain is: Lys Asp Lys Ile Gly Lys,  
3 corresponding to amino acids 40 to 45 of the protein.  
4

5 The H epitope for the Simian virus 40 VP1 protein  
6 is: Asp Asp Ser Pro Asp Lys Glu, corresponding to amino acids  
7 77 to 83 of the protein (two adjacent and overlapping H epitopes).  
8

9 The H epitope for the available sequence of the  
10 fiber protein of adenovirus type 2 (N-terminal 80%) is: Asn  
11 Lys Asn Asp Asp Lys, corresponding to amino acids 393 to  
12 398 of the protein.  
13

14 The H epitope of the Sindbis virus membrane  
15 glycoprotein E1 is: Ser Asp Arg Glu Gly Gln corresponding  
16 to amino acids 322 to 327.  
17

18 The H epitope of the Sindbis virus membrane  
19 glycoprotein E2 corresponds to the following amino acid  
20 chain: Asp Glu Ala Asp Asp Asn corresponding to amino acids  
21 36 to 41.  
22

23 The H epitope for the Sindbis virus membrane  
24 glycoprotein E3 corresponds to amino acids 27 to 32 and  
25 has the following sequence: Thr Arg Glu Pro Ser Arg.  
26

27 The H epitope for the foot and mouth disease  
28 virus capsid protein VP1 corresponds to amino acids 179 to  
29 184 and has the following amino acid sequence: Arg Met Lys  
30 Arg Ala Glu.

1        There are two sequences of amino acids for the influenza  
2        hemagglutinin antigen (Japan strain) which determine H-epitopes  
3        of equivalent hydrophilicity i.e., they provide identical  
4        local average hydrophilicity. They are Glu Lys Glu Asn  
5        Pro Arg (correspond to amino acids 96-101) and Lys Glu  
6        Asn Pro Arg Asp (correspond to amino acids 97 - 102). Similar-  
7        ly, the sequence of amino acids for the influenza hemagglutinin  
8        antigen (Victoria A strain) which determine the H-epitope  
9        is: Asn Asp Asn Ser Asp Lys (corresponding to amino acids  
10       188 - 193).

11  
12       Similarly, there are two sequences of amino acids for  
13       the Fowl Plague virus hemagglutinin antigen which determine  
14       H-epitopes of identical local average hydrophilicity. They  
15       are: Glu Arg Arg Glu Gly Asn (corresponding to amino acids  
16       97 - 102) and Arg Arg Glu Gly Asn Asp (corresponding to  
17       amino acid 98 - 103).

18  
19       Similarly, the sequence of amino acids for the human  
20       chorionic Gonadotropin B subunit antigen which determine the  
21       H-epitope is: Arg Arg Ser Thr Thr Asp corresponding to  
22       amino acids 94 - 99.

23  
24       Similarly, the sequence of amino acids for the Human  
25       Beta-2 microglobulin antigen which determines the H-epitope  
26       is: Pro Thr Glu Lys Asp Glu which corresponds to amino acids  
27       73-78.

28  
29       Similarly, the sequence of amino acids for the human  
30       Myelin basic protein antigen which determines the H-epitope.

1 is: Gly Arg Asp Ser Arg Ser corresponding to amino acids  
2 159 - 164.

3  
4 Similarly, the sequence of amino acids for the Cholera  
5 Toxin B-chain antigen which determines the H-epitopes  
6 is: Glu Ala Lys Val Glu Lys corresponding to amino acids  
7 79 - 84.

8  
9 Another hepatitis B surface antigen has been studied to  
10 determine its sequence of six amino acids which determine the  
11 H-epitope. Its sequence is: Lys Pro Ser Asp Gly Asn  
12 corresponding to amino acid 141 - 146.

13  
14 The sequence of amino acid for the E. Coli Heat Labile  
15 Toxin which determine the H-epitope is Glu Arg Met Lys Asp  
16 Thr corresponding to amino acids 66 - 71.

17  
18 The sequence of amino acids for the E. Coli Heat  
19 Stable Toxin provides two identical H-epitopes whose amino  
20 acid sequence is Asp Ser Ser Lys Glu Lys and Ser Glu Lys Lys  
21 Ser glu corresponding to amino acids 26 - 31 and 46 - 51,  
22 respectively.

23  
24 The ragweed allergen Ra3 has an H-epitope whose amino  
25 acid sequence is Cys Thr Lys Asp Gln lys corresponding to  
26 amino acid 88 - 93.

27  
28 The ragweed allergen Ra5 has an H-epitope whose amino  
29 acid sequence is Ser Lys Lys Cys Gly Lys corresponding to amino  
30 acids 40 - 45.

- 28 -

1       The streptococcal M protein (strain 24) has two identical H-epitopes whose amino acid sequences are

Arg Lys Ala Asp Leu Glu and

Lys Ala Asp Leu Glu Lys

5       corresponding to amino acids 58-63 and 59-64.

The trypanosoma brucei variant surface glycoprotein 117 has an H-epitope whose amino acid sequence is

Lys Ala Lys Glu Lys Gly

10       corresponding to amino acids 50-55.

In preparing vaccines according to the invention, it is preferred to attach to the six amino acids which define the H-epitope at least three amino acids on either side thereof:

15       These three amino acids can be the same acids in the same sequences as they occur in the natural protein. However, other acids can also be used. For instance, in the hepatitis Bs vaccine the amino acid sequence can be

Aba Aba Thr Lys Pro Thr Asp Gly Asn Aba Thr Aba

20       (Aba residues have replaced Cys residues).

The synthetic vaccines are prepared as follows:

1. Chemical Synthesis: The Merrifield solid phase procedure is used to build up the appropriate sequence of L-amino acids from the carboxyl terminal amino acid to the amino terminal acid and to add the fatty acid moiety(s) at the N-terminus. Starting with appropriate carboxyl terminal amino acid attached to a polystyrene (or other appropriate) resin via chemical linkage to a chloromethyl group of the resin, amino acids are added one by one using the following procedure for each:

25

30

- 29 -

- 1 a) Peptidyl resin is washed with methylene chloride,
- b) neutralized by mixing for 10 min. at room temperature  
with 5% (v/v) diisopropylethylamine (or other hindered  
base) in methylene chloride,
- 5 c) washed with methylene chloride.
- d) An amount of amino acid or fatty acid equal to six  
times the molar amount of the growing peptide chain is  
activated by combining it with one-half as many moles  
of a carbodiimide (e.g. dicyclohexylcarbodiimide, di-  
isopropylcarbodiimide) for 10 minutes at 0 °C to form  
10 the symmetric anhydride of the amino acid or fatty  
acid. The amino acid used should be provided originally  
as the N- $\alpha$ -butyl-oxycarbonyl derivative, with side  
chains protected with benzyl esters (aspartic and glu-  
15 tamic acids), benzyl ethers (serine, threonine,  
cysteine, tyrosine), benzyl oxycarbonyl groups (lysine)  
or other protecting groups commonly used in peptide  
synthesis. Fatty acids require no protecting groups.
- e) The activated amino acid or fatty acid is reacted with  
20 the peptide resin for 2 hours at room temperature, re-  
sulting in addition of the new amino acid or fatty acid  
to the end of the growing peptide chain.
- f) The resin is washed with methylene chloride.
- g) The N- $\alpha$ -butylocarbonyl group is removed from the most  
25 recently added amino acid by reacting with 30% (v/v)  
trifluoroacetic acid in methylene chloride for 30 minutes  
at room temperature.
- h) The resin is washed with methylene chloride.
- 30 i) Steps a through h are repeated until the required  
peptide sequence has been constructed, with the fatty  
acid being put in place last.

1 The peptide is then removed from the resin, and simultaneously  
2 the side-chain protecting groups are removed, by reacting with  
3 anhydrous hydrofluoric acid containing 10% (v/v) of anisole.  
4 Subsequently, the peptide can be purified by gel filtration,  
5 ion exchange or high pressure liquid chromatography, or other  
6 suitable means.

7 In some cases, chemical synthesis can be carried out  
8 without the solid phase resin, in which case the synthetic  
9 reactions are performed entirely in solution. The reactions,  
10 and the final product, are otherwise essentially identical.

11 The synthetic vaccines of the invention can readily  
12 be incorporated into compositions possessing an oily adjuvant  
13 such as Freund's adjuvant (complete or incomplete) or into  
14 liposomes due to their lipophilic nature which causes them to  
15 be retained in these adjuvants longer. This longer retention  
16 time facilitates the desired immune response.

17 When two fatty acid or similar moieties are desired  
18 as carriers for the peptide, it is preferred that the synthetic  
19 vaccine be formed using the following technique. Thus, the  
20 N-terminal end of the peptide, while still on the Merrifield  
21 resin, is coupled to a lysine moiety or other suitable linking  
22 bridge possessing diaminoalkylene or-alkenylene moiety. The  
23 lysine, which contains two amino groups, is linked to the N-  
24 terminal end of the peptide using, for instance, bis-tertiary  
25 butyloxycarbonylated lysine as reactant as set forth above.  
26 After deprotection, both the alpha and the epsilon amino groups  
27 are available for fatty acid coupling. Treatment as above  
28 yields the fatty acid disubstituted lysyl peptide, which  
29  
30

1 thereafter can be cleaved from the resin with hydrofluoric acid.  
2 This derivative should be retained to a greater extent by  
3 Freund's adjuvant or liposome, and form more stable self-  
4 aggregates.

5 A simple variation of this approach can be used to  
6 place one (or more) fatty acids at the C-terminus of the peptide.  
7 A lysine residue can be coupled to the Merrifield resin as the  
8 first step of the synthesis. This lysine can be differentially  
9 protected, for example, as the alpha-tertiary butyloxycarbonyl,  
10 epsilon-9-fluorenylmethyloxycarbonyl derivative, allowing select-  
11 ive deprotection of the epsilon amino group by treatment with  
12 piperidine/methylene chloride (1:1) at 25°C for 30 minutes. A  
13 fatty acid or other lipophilic substance may then be coupled  
14 by the same procedure used in coupling fatty acids to the N-  
15 terminus of the peptide. Thereafter, the alpha-tertiary butyl-  
16 oxy carbonyl group is removed by the usual acid treatment, and  
17 peptide synthesis is completed by the usual procedures. If two  
18 or more alpha-tertiary butyloxycarbonyl, epsilon-9-fluorenyl-  
19 methyloxycarbonyl lysines are sequentially treated as above, a  
20 product can be generated that bears multiple fatty acid substi-  
21 tuted lysines at its C-terminus.

22 It is possible, by the procedures described above, to  
23 make antigenic peptide - fatty acid conjugates wherein there ex-  
24 ist one or multiple fatty acid residues at the N-terminus of the  
25 peptide, at the C-terminus of the peptide, or at both ends sim-  
26 ultaneously. Although lysine is used in the example above, it  
27 is understood that any diamino acid could be used, including  
28 such amino acids as ornithine, or alpha-, gamma-diamino butyric  
29 acid.  
30



- 32 -

1 In order to more fully illustrate the invention and  
2 the manner of practicing the same, the following examples are  
3 presented.

4 EXAMPLE 1

5 Coupling of a palmityl moiety to Glycyl hepatitis B antigenic  
6 peptide (H peptide).

7 An initial acid solution comprising 3 parts tri-  
8 fluoroacetic acid and 7 parts methylene chloride was formed.  
9 There was also formed a base solution comprising 5 parts  
10 diisopropylethylamine and 95 parts methylene chloride.  
11 Merrifield resin was used as starting material. Specifically  
12 the starting material was commercially available tertiary  
13 butyloxycarbonylated glycyl resin ester containing 0.33 m moles  
14 of glycine per gram of resin.

15 Glycyl H peptidyl resin. The H peptide was con-  
16 structed on 1 gram of butyloxycarbonylated glycyl resin by  
17 the classical Merrifield method. Its structure was Gly Gly  
18 Gly Aba Aba Thr Lys Pro Thr Asp Gly Asn Aba Thr Aba Gly Resin,  
19 with amino acid side chains protected as follows: Thr benzyl  
20 ether, Lys carbobenzoxy amide, and Asp benzyl ester.

21 In this synthesis, an N-terminal Gly Gly Gly sequence  
22 was added to act as a spacer to separate the palmityl moiety  
23 from the rest of the peptide. Palmitic acid was coupled to  
24 the N-terminal glycyl residue as follows:

25 1. The N-terminal tertiary butyloxycarbonyl group  
26 was removed by 30 min (25°C) treatment with acid solution  
27 as in the Merrifield procedure.

28 2. The newly exposed alpha amino group was  
29 neutralized by treatment with base solution for 10 min (25°C),  
30 again, as in the Merrifield procedure.

1           3. Three equivalents (1 m mole) of palmitic  
2 anhydride was dissolved in 15 ml of methylene chloride, and  
3 reacted with the peptidyl resin. The reaction was complete  
4 after 2 hrs. (25°C) as determined by the Kaiser ninhydrin  
5 test, which became negative at that time.

6           4. The palmityl H peptide was cleaved from the  
7 resin, and all blocking groups were removed, by treatment  
8 with hydrofluoric acid/anisole (9/1) for 15 minutes at 0°C.  
9 The palmityl H peptide was extracted from the resin beads by  
10 sequential washing with glacial acetic acid, acetic acid/water  
11 (50/50), and with water. Washes were pooled and lyophilized.

12           5. The crude product was purified by extraction  
13 with  $\text{MeCl}_2$  to remove traces of anisole and lipid contaminants.

14           6. The product was dissolved in glacial acetic  
15 acid, although it is preferred to employ dimethylsulfoxide,  
16 then diluted with 9 parts water. This causes the immediate  
17 formation of a microemulsion of the peptide, which can be  
18 observed as an opalescent appearance of the solution. This  
19 material was lyophilized, and resuspended in phosphate buffered  
20 saline for immunizations.

## 21 22 EXAMPLE 2

23           Coupling of two palmityl moieties to Lysyl hepatitis B antigenic  
24 peptide (H peptide).

25           Lysyl H peptidyl resin: This peptide was constructed  
26 on 1 gram of tertiary butyloxycarbonylated glycyl resin by  
27

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the Merrifield method. Its structure was: Lys Gly Gly Aba :  
Aba Thr Lys Pro Thr Asp Gly Asn Aba Thr Aba Gly Resin, with  
amino acid side chains protected as follows: Thr benzyl ether,  
N-terminal Lys alpha and epsilon tertiary butyloxycarbonyl,  
central Lys epsilon carbobenzoxy amide, and Asp benzyl ester.

Palmitic acid was coupled to the alpha and epsilon  
amino groups of the N-terminal lysyl residue as follows:

1. The N-terminal tertiary butyloxycarbonyl  
and epsilon tertiary butyloxycarbonyl groups were removed by  
30 min. (25°C) treatment with acid solution as in the Merri-  
field procedure.

2. The newly exposed alpha and epsilon amino  
groups were neutralized by treatment with base solution for  
10 min. (25°C).

3. Three equivalents (1 m mole) of palmitic  
anhydride were dissolved in 15 ml of  $\text{MeCl}_2$ , and reacted with  
the peptidyl resin. The reaction was stopped after 2 hours  
(25°C) but was only about 80% complete as determined by the  
Kaiser ninhydrin test. Therefore, a second coupling under  
identical conditions was carried out. After this coupling,  
the ninhydrin test was negative, indicating complete coupling  
of palmitic acid to both the alpha and epsilon amino groups  
of the N-terminal lysyl residue.

4. The product was cleaved from the resin and  
purified as in steps 4 through 6 of Example 1.

5. The results of a preliminary immunization study  
using rabbits are shown in Table I. These results show that  
the conjugate is capable of producing anti-HbsAg responses with

or without use of an adjuvant, although the highest titers were obtained with adjuvant present.

TABLE I

Rabbit I.D. #	Titer (days after initial inoculation)			
	Pre-immune	20	37	54
657	0	0	1.9	41.5
658	2.8	74.2	-	-
659	3.0	3.3	52.0	52.3
661	5.2	4.5	11.0	15.1
662	0.6	0.6	2.9	2.9

Immunizations were given on days 1 and 23. These consisted of 0.5 ml of Freund's complete adjuvant emulsified in 0.5 ml of phosphate-buffered saline for rabbits 657-659, and 1 ml of phosphate-buffered saline without adjuvant for 661 and 662. Each dose contained 0.05 mg dipalmityl H peptide on day 1 and 0.2 mg on day 23. Titers were determined by the Ausab test (Abbott Laboratories); titers over 10.0 were commonly considered to represent immunity to type B hepatitis.

The data above reveals that the antibody titer of test animals is dramatically increased when a synthetic vaccine of the invention containing an alkyl or alkenyl carrier is administered to the host animal.

1 By the procedure of the invention there is realized a  
2 vaccine which is characterized by the absence of an amino acid  
3 sequence of the entire protein antigen or allergen. For  
4 instance, in the case of a hepatitis B vaccine, the vaccine  
5 is free of other peptide sequences of the hepatitis B surface  
6 antigen protein, or other proteins found in the virion. Vac-  
7 cines can be synthesized which are free of biologically produced  
8 components, free of viral components whether they be active or  
9 inactive, free of antibodies, free of deoxyribonucleic acid  
10 (DNA) and free of lipids, and are therefore likely to be sub-  
11 stantially free from undesirable side effects commonly found with  
12 other vaccines (unintentional infection with virus, allergic re-  
13 action, fevers, etc.).

14  
15 The synthetic vaccines are characterized by exceptional  
16 specificity and evoke an unusual and special response when intro-  
17 duced into a host animal. Whereas a vaccine made of natural mat-  
18 erial and introduced into a host animal usually evokes an  
19 immunological response by the creation of antibodies specific  
20 to a number of distinct epitopes present on the antigens found  
21 in that vaccine, when the vaccine of the present invention is  
22 introduced into a host animal, it causes the formation of anti-  
23 bodies which are mono-specific, i.e., are specific to the single  
24 antigenic site on the vaccine. Thus, the vaccines of the pre-  
25 sent invention can be employed to form immune globulin comprising  
26 a mono-specific antibody. These mono-specific antibodies may be  
27 produced in animals, to serve as a source for diagnostic immuno-  
28 globulin to be used in serological testing, for example in ident-  
29 ifying strain types of pathogenic organisms isolated from  
30 infected individuals.

1 In the preparation of a vaccine the concentration of the  
2 same in the physiologically acceptable medium will vary depending  
3 on the number and types of H epitopes contained therein.

4 Generally speaking, the active component of the vaccine can be  
5 present in a concentration which is lower than the concentration  
6 of active material in known vaccines since in the known vaccines  
7 higher concentrations were required in order to have present  
8 the required number of antigenic determinants to evoke the de-  
9 sired immunological response. The vaccine concentration will,  
10 of course, vary from vaccine to vaccine. Generally speaking,  
11 its concentration will be from 5 to 100 u gm, preferably 20 to  
12 50 u gm per dose to give suitable immunogenicity. It is partic-  
13 ularly contemplated to use the vaccine in a dosage of .01 to  
14 100, especially .01 to 10 micrograms per dose.

15  
16 The vaccine will have sufficient potency to provide an  
17 antibody titer of at least 1:100 when determined by tests such  
18 as passive hemagglutination. For instance, the vaccines of  
19 hepatitis B<sub>s</sub> have an antibody HB<sub>s</sub> titer of at least 1:100 when  
20 determined by passive hemagglutination (standardized by tests on  
21 a frozen anti-serum control) in at least four chimpanzees  
22 immunized with two doses of the standard vaccine in accordance  
23 with the recommended schedule, the anti- HB<sub>s</sub> remaining detectable  
24 at a titer greater than 1:10 for at least a year following the  
25 onset of immunization of the chimpanzees. Naturally, the vaccine  
26 concentration can vary from these concentrations depending upon  
27 the effect desired.

28  
29 The vaccine can be administered by subcutaneous or intra-  
30

1 muscular injection. While the preferred route would depend  
2 upon the particular vaccine, it is believed that intramuscular  
3 injection will be generally suitable. Frequency of adminis-  
4 tration will vary depending upon the vaccine and the nature and  
5 type of epitopes and their concentration in the active compo-  
6 nent. Generally speaking, the vaccine will be administered in  
7 two doses about one month apart followed by a booster at six  
8 months to one year after primary immunization. Of course, the  
9 dosage will depend upon the size of the host animal being  
10 inoculated. The subsequent doses or the booster will depend  
11 on the level of antibody in the blood as a result of the initial  
12 immunization. Licensable adjuvants conventionally employed in  
13 vaccine manufacture can be utilized.

14  
15 In the case of a hepatitis vaccine as particularly con-  
16 templated herein, the same is recommended for all persons at  
17 risk of developing hepatitis B infection and particularly those  
18 at especially high risk such as patients and staff on hemodial-  
19 ysis unit, medical personnel, persons of tropical populations  
20 and those visiting the tropics. In the case of tropical popu-  
21 lations, particularly in Africa, Asia, the Mediterranean region  
22 and South America, where high incidence of hepatitis B infections  
23 has been consistently observed, the vaccine should be adminis-  
24 tered sufficiently early in life to prevent acquisition of chron-  
25 ic carrier state infection which tend to occur in these regions  
26 within the first five years of life. In fact, the vaccine is  
27 useful for all persons not already protected against hepatits  
28 B infections as a result of prior immunity.

1 WHAT IS CLAIMED IS:

2 1. A synthetic vaccine comprising a peptide residue  
3 coupled to one or more alkyl or alkenyl groups of at least 12  
4 carbon atoms or other lipophilic substance wherein said peptide  
5 residue contains a sequence of 6 amino acids corresponding to  
6 the sequence of such amino acids in a protein antigen or aller-  
7 gen where the greatest local average hydrophilicity of the anti-  
8 gen or allergen is found, said local hydrophilicity of said  
9 protein antigen or allergen determined by:

10 A. assigning relative hydrophilicity values to  
11 the amino acids of the protein antigen or  
12 allergen in accordance with the relative  
13 relationship of such amino acids as shown  
14 in the table below:

15 TABLE 1

16 <u>Amino Acid</u>	16 <u>Hydrophilicity Value</u>
17 Arginine	3.0
18 Aspartic Acid	3.0 $\pm$ 1
19 Glutamic Acid	3.0 $\pm$ 1
20 Lysine	3.0
21 Serine	0.3
22 Asparagine	0.2
23 Glutamine	0.2
24 Glycine	0.0
25 Proline	-0.5 $\pm$ 1
26 Threonine	-0.4
27 Alanine	-0.5
28 Histidine	-0.5
29 Cysteine	-1.0
30 Methionine	-1.3
31 Valine	-1.5



1	Isoleucine	-1.8
2	Leucine	-1.8
3	Tyrosine	-2.3
4	Phenylalanine	-2.5
5	Tryptophan	-3.4

6  
7 B. determining the repetitive local average of  
8 hydrophilicity values at a plurality of  
9 points along the amino acid chain;

10 C. determining from such points of repetitive  
11 averages the points of greatest local average  
12 hydrophilicity

13 said vaccine when free of an amino acid sequence corresponding  
14 to the entire protein antigen or allergen evoking a protective  
15 immunological response by stimulation of antibody formation  
16 against antigen or allergen when introduced into a host animal.

17  
18 2. A vaccine according to claim 1, wherein  
19 said chain of amino acids comprises no more than 50 amino acids.

20  
21 3. A vaccine according to claim 2, wherein  
22 said chain of amino acids comprises no more than 40 amino acids.

23  
24 4. A vaccine according to claim 2, wherein  
25 said chain of amino acids comprises no more than 30 amino acids.

26  
27 5. A vaccine according to claim 2, wherein  
28 said chain of amino acids comprises no more than 20 amino acids.

29  
30

1                   6. A vaccine according to claim 5, wherein  
2 said chain of amino acids comprises no more than 18 amino acids.

3  
4                   7. A vaccine according to claim 2, wherein  
5 said chain of amino acids comprises no more than 14 amino acids.

6  
7                   8. A vaccine according to claim 2, wherein  
8 said chain of amino acids comprises 12 - 18 amino acids.

9  
10                  9. A vaccine according to claim 5, wherein  
11 said peptide residue is coupled to an alkyl or alkenyl group  
12 of 12 - 36 carbon atoms.

13  
14                  10. A synthetic vaccine according to claim  
15 5, wherein said peptide residue is coupled to an alkyl or alkenyl  
16 group of 12 - 24 carbon atoms.

17  
18                  11. A synthetic vaccine according to claim  
19 9, wherein said alkyl or alkenyl group is coupled to the terminal  
20 amino group of said peptide residue via a carbonyl group.

21  
22                  12. A vaccine according to claim 9, wherein  
23 said alkyl or alkenyl group is coupled directly to the terminal  
24 amino group of said peptide residue.

25  
26                  13. A vaccine according to claim 1, wherein  
27 said peptide residue is coupled to a lipophilic substance.

28  
29                  14. A vaccine according to claim 14, wherein  
30

1 said lipophilic substance is

2 palmitic acid

3 stearic acid

4 behenic acid

5 oleic acid

6 mycolic acid

7  
8 15. A synthetic vaccine according to claim 5,  
9 which is a vaccine for hepatitis B, said vaccine containing a  
10 peptide residue having the following sequence of amino acids:  
11 Lys Pro Thr Asp Gly Asn.

12  
13 16. A synthetic vaccine according to claim 15,  
14 wherein said sequence of amino acids comprises the following  
15 sequence: Aba Aba Thr Lys Pro Thr Asp Gly Asn Aba Thr Aba.

16  
17 17. A synthetic vaccine according to claim 15,  
18 wherein said sequence of amino acids comprises the following  
19 sequence: Cys Cys Thr Lys Pro Thr Asp Gly Asn Cys Thr Cys.

20  
21 18. A synthetic vaccine according to claim 15,  
22 characterized by the absence of other peptide sequences of the  
23 hepatitis B virion.

24  
25 19. A vaccine according to claim 1, which is  
26 free of active or inactive viral contaminants.

27  
28 20. A vaccine according to claim 1, which is  
29 substantially free of biologically produced components.

30  
31 21. A vaccine according to claim 15, which is

1 free of hepatitis B antibodies.

2 22. A vaccine according to claim 15, which is  
3 free of DNA or RNA.  
4

5 23. A vaccine according to claim 15, which is  
6 free of lipids, but contains other lipophilic components.  
7

8 24. A vaccine according to claim 5, comprising a  
9 peptide residue having the following sequence of amino acids:  
10 Pro Arg Glu Glu Pro Arg.  
11

12 25. A vaccine according to claim 5, comprising  
13 a peptide residue having the following sequence of amino acids:  
14 Val Glu Arg Ser Lys Ala.  
15

16 26. A vaccine according to claim 5, comprising  
17 a peptide residue having the following sequence of amino acids:  
18 Lys Arg Gly Pro Asp Ser.  
19

20 27. A vaccine according to claim 5, comprising  
21 a peptide residue having the following sequence of amino  
22 acids: Arg Asn Val Pro Gly Lys.  
23

24 28. A vaccine according to claim 5, comprising  
25 a peptide residue having the following sequence of amino  
26 acids: Arg Gly Arg Pro Lys Glu Lys.  
27

28 29. A vaccine according to claim 5, comprising  
29  
30

1 a peptide residue having the following sequence of amino acids:  
2 Glu Thr Arg Lys Arg.

3

4 30. A vaccine according to claim 5, comprising  
5 a peptide residue having the following sequence of amino acids:  
6 Leu Arg Glu Ile Gle Arg Leu.

7

8 31. A vaccine according to claim 5, comprising  
9 a peptide residue having the following sequence of amino acids:  
10 Lys Ser Lys Pro Lys Asp.

11

12 32. A vaccine according to claim 5, comprising  
13 a peptide residue having the following sequence of amino acids:  
14 Lys Asp Lys Ile Gly Lys.

15

16 33. A vaccine according to claim 5, comprising  
17 a peptide residue having the following sequence of amino acids:  
18 Asp Asp Ser Pro Asp Lys Glu.

19

20 34. A vaccine according to claim 5, comprising  
21 a peptide residue having the following sequence of amino acids:  
22 Asn Lys Asn Asp Asp Lys.

23

24 35. A vaccine according to claim 5, comprising  
25 a peptide residue having the following sequence of amino acids:  
26 Ser Asp Arg Glu Gly Gln.

27

28 36. A vaccine according to claim 5, comprising  
29 a peptide residue having the following sequence of amino acids:  
30 Asp Glu Ala Asp Asp Asn.

1 37. A vaccine according to claim 5 comprising  
2 a peptide residue having the following sequence of amino acids:  
3 Thr Arg Glu Pro Ser Arg.

4  
5 38. A vaccine according to claim 5, comprising  
6 a peptide residue having the following sequence of amino acids:  
7 Arg Met Lys Arg Ala Glu.

8  
9 39. A vaccine according to claim 5, comprising  
10 a peptide residue having the following sequence of amino acids:  
11 Glu Lys Glu Asn Pro Arg.

12  
13 40. A vaccine according to claim 5, comprising  
14 a peptide residue having the following sequence of amino acids:  
15 Lys Glu Asn Pro Arg Asp.

16  
17 41. A vaccine according to claim 5, comprising  
18 a peptide residue having the following sequence of amino acids:  
19 Asn Asp Asn Ser Asp Lys.

20  
21 42. A vaccine according to claim 5, comprising  
22 a peptide residue having the following sequence of amino acids:  
23 Glu Arg Arg Glu Gly Asn.

24  
25 43. A vaccine according to claim 5, comprising  
26 a peptide residue having the following sequence of amino acids:  
27 Arg Arg Glu Gly Asn Asp.

28  
29 44. A vaccine according to claim 5, comprising  
30

1 a peptide residue having the following sequence of amino acids:

2 Arg Arg Ser Thr Thr Asp.

3

4 45. A vaccine according to claim 5, comprising  
5 a peptide residue having the following sequence of amino acids:

6 Pro Thr Glu Lys Asp Glu.

7

8 46. A vaccine according to claim 5, comprising  
9 a peptide residue having the following sequence of amino acids:

10 Gly Arg Asp Ser Arg Ser.

11

12 47. A vaccine according to claim 5, comprising  
13 a peptide residue having the following sequence of amino acids:

14 Glu Ala Lys Val Glu Lys.

15

16 48. A vaccine according to claim 5, comprising  
17 a peptide residue having the following sequence of amino acids:

18 Lys Pro Ser Asp Gly Asn.

19

20 49. A vaccine according to claim 5, comprising  
21 a peptide residue having the following sequence of amino acids:

22 Glu Arg Met Lys Asp Thr.

23

24 50. A vaccine according to claim 5, comprising  
25 a peptide residue having the following sequence of amino acids:

26 Asp Ser Ser Lys Glu Lys.

27

28 51. A vaccine according to claim 5, comprising  
29 a peptide residue having the following sequence of amino acids:

30 Ser Glu Lys Lys Ser Glu.

1 52. A vaccine according to claim 5, comprising  
2 a peptide residue having the following sequence of amino acids:  
3 Cys Thr Lys Asp Gln Lys.  
4

5 53. A vaccine according to claim 5, comprising  
6 a peptide residue having the following sequence of amino acids:  
7 Ser Lys Lys Cys Gly Lys.  
8

9 54. A vaccine according to claim 5, comprising  
10 a peptide residue having the following sequence of amino acids:  
11 Arg Lys Ala Asp Leu Glu.  
12

13 55. A vaccine according to claim 5, comprising  
14 a peptide residue having the following sequence of amino acids:  
15 Lys Ala Asp Leu Glu Lys.  
16

17 56. A vaccine according to claim 5, comprising  
18 a peptide residue having the following sequence of amino acids:  
19 Lys Ala Lys Glu Lys Gly.  
20

21 57. A vaccine according to claim 5, comprising  
22 a peptide residue having the following sequence of amino acids:  
23 Glu Leu Val Arg Lys Arg Glu Glu Cys Leu Asp Ala.  
24

25 58. A vaccine according to claim 5, comprising  
26 a peptide residue having the following sequence of amino acids:  
27 Thr Val Phe Lys Asp Gly Asp Glu Ala Glu Asp Phe.  
28

29 59. A vaccine according to claim 5, comprising  
30



1 a peptide residue having the following sequence of amino acids:

2 His Asp Phe Arg Ser Asp Glu Ile Glu His Leu Val.

3

4 60. A vaccine according to claim 5, comprising

5 a peptide residue having the following sequence of amino acids:

6 Met Ala Gly Asp Pro Arg Tyr Glu Glu Ser Leu His.

7

8 61. A vaccine according to claim 5, comprising

9 a peptide residue having the following sequence of amino acids:

10 Ile Phe Thr Asn Ser Arg Gly Lys Arg Ala Ser Lys.

11

12 62. A vaccine according to claim 5, comprising

13 a peptide residue having the following sequence of amino acids:

14 Thr Thr Phe Lys Arg Lys His Phe Arg Pro Thr Pro.

15

16 63. A vaccine according to claim 5, comprising

17 a peptide residue having the following sequence of amino acids:

18 Arg Thr Val Lys Thr Thr Lys Glu Ser Leu Val Ile.

19

20 64. A composition comprising a synthetic

21 antigen or allergen of the formula:

22

23

24

25

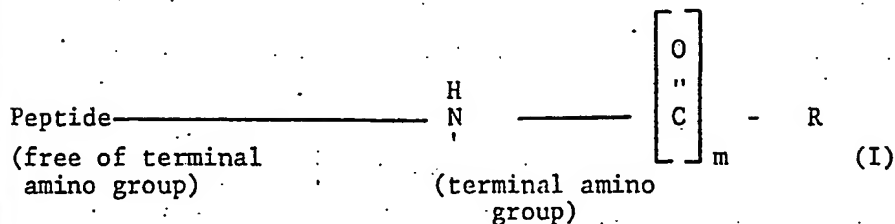
26

27

28

29

30



wherein

- 49 -

1  $m = 0$  or 1

2 R is a substituted or unsubstituted alkyl or alkenyl  
3 radical of at least 12 carbon atoms and

4 Peptide is a residue containing a sequence of 6  
5 amino acids corresponding to the sequence of  
6 such amino acids in a protein antigen or  
7 allergen where the greatest local average  
8 hydrophilicity of the antigen or allergen  
9 is found, said local hydrophilicity of said  
10 protein antigen or allergen determined by:

11 A. assigning relative hydrophilicity values to  
12 the amino acids of the protein antigen or  
13 allergen in accordance with the relative  
14 relationship of such amino acids as shown in  
15 the table below:

TABLE I

Amino Acid	Hydrophilicity Value
Arginine	3.0
Aspartic Acid	3.0 $\pm$ 1
Glutamic Acid	3.0 $\pm$ 1
Lysine	3.0
Serine	0.3
Asparagine	0.2
Glutamine	0.2
Glycine	0.0
Proline	-0.5 $\pm$ 1
Threonine	-0.4
Alanine	-0.5
Histidine	-0.5
Cysteine	-1.0
Methionine	-1.3
Valine	-1.5
Isoleucine	-1.8
Leucine	-1.8

1	Tyrosine	-2.3
2	Phenylalanine	-2.5
3	Tryptophan	-3.4

4           B: determining the repetitive local average of  
5           hydrophilicity values at a plurality of  
6           points along the amino acid chain;

7           C. determining from such points of repetitive  
8           averages the points of greatest local average  
9           hydrophilicity,

10 said synthetic antigen when free of an amino acid sequence  
11 corresponding to the entire protein antigen or allergen evoking  
12 a protective immunological response by stimulation of antibody  
13 formation against an antigen or allergen when introduced into a  
14 host animal.

15  
16                   65. A composition according to claim 64,  
17 wherein R is alkyl or alkenyl of up to 36 carbon atoms.

18  
19                   66. A composition according to claim 65,  
20 wherein R is alkyl or alkenyl of up to 18 carbon atoms.

21  
22                   67. A composition according to claim 66,  
23 wherein R is alkyl or alkenyl of up to 12 carbon atoms.

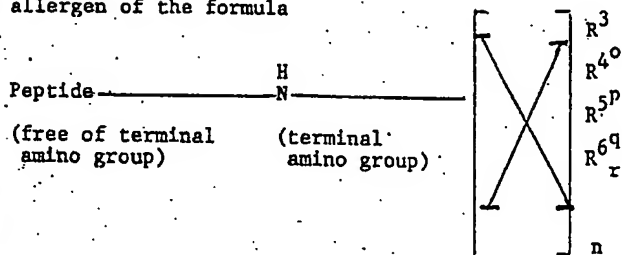
24  
25                   68. A composition according to claim 64,  
26 wherein m is 0.

27  
28                   69. A composition according to claim 64,  
29 wherein m is 1.

30

- 51 -

70. A composition comprising a synthetic antigen or allergen of the formula



wherein

peptide has the same meaning given in claim 1;

$R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$  are each  $C_{12}$ - $C_{36}$  alkyl or alkenyl

which may be straight or branched chained and

substituted or unsubstituted;

$o$ ,  $p$ ,  $q$ , and  $r$  are at each 0 or 1 and the sum of

$o$ ,  $p$ ,  $q$ , and  $r$  is equal to  $n$ ;

$n$  is 2 - 4;

$X$  is a polyfunctional group having 3 to 5 functional

groups, at least one of which is bound to said

terminal amino group, and at least one of said

function groups bound to one of  $R^3$ ,  $R^4$ ,  $R^5$  or  $R^6$ .

71. A synthetic vaccine or antigen according to claim 6, wherein  $X$  is

lysine

ornithine

$\alpha$ , $\gamma$ -diamino butyric acid

72. A synthetic vaccine or antigen according to claim 71, wherein X comprises a carbonyl group bonded to the residue of said terminal amine group and at least one amido group bonded to at least one of  $R^3$ ,  $R^4$ ,  $R^5$  or  $R^6$ .

73. A synthetic vaccine or antigen according to claim 72, wherein X in turn is representable by the formula:



wherein

A is a bifunctional group one end of which is linked to M and the other end of which is linked to the terminal amino group of the synthetic peptide;

M is alkylene or alkenylene of 2 to 5 C atoms;

B, C, D and E are each bifunctional groups one end of which is linked to M and the other end of which is linked to a  $C_{12}$ - $C_{36}$  alkyl or alkenyl group and

b, c, d and e are each 1 or 0 and the sum of b, c, d and e is 2 - 4.

74. A synthetic vaccine or antigen according to claim 6, wherein at least one alkyl or alkenyl moiety is attached to the C-terminal carboxyl group or to a side chain reactive group of said peptide residue.

1 75. A vaccine as in claim 1, wherein a fatty acid or  
2 other lipophilic substance is covalently bound to the side  
3 chain amino group of a diamino acid residue at the C-terminus of  
4 the peptide moiety.

5  
6 76. A vaccine as in claim 1, wherein fatty acids or  
7 other lipophilic substances are covalently bound to the side  
8 chain amino groups of several diamino acid residues at the C-  
9 terminus of the peptide moiety.

10  
11 77. A vaccine as in claim 75, wherein a fatty acid or  
12 other lipophilic substance is also covalently bound to the N-  
13 terminal amino group, and/or the side chain amino group of an N-  
14 terminal diamino acid residue.

15  
16 78. A vaccine as in claim 76, wherein fatty acids or  
17 other lipophilic substances are also covalently bound to the N-  
18 terminal amino group, and/or the side chain amino groups of  
19 several N-terminal diamino acid residues.  
20  
21  
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28  
29  
30